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Accurate Assignment of Ethanol Origin in Postmortem Urine: A Case Study

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16. Abstract

Specimens from fatal aviation accident victims are submitted to the FAA Civil Aerospace Medical Institute for toxicological analysis. During toxicological evaluations, ethanol analysis is performed on all cases. Care must be taken when interpreting a positive ethanol result due to the potential for postmortem ethanol formation. Several indicators of postmortem ethanol formation exist; however, none are completely reliable. The consumption of ethanol has been shown to alter the concentration of two major serotonin metabolites, 5-hydroxytryptophol (5-HTOL) and 5-hydroxyindole-3-acetic acid (5-HIAA). While the 5-HTOL/5-HIAA ratio is normally very low, previous studies using living subjects have demonstrated that the urinary 5-HTOL/5-HIAA ratio is significantly elevated for 11-19 hours after acute ethanol ingestion. Recently, our laboratory developed and validated an analytical method for the simultaneous determination of both 5-HTOL and 5-HIAA in forensic urine samples using a simple liquid/liquid extraction and LC/MS/MS and LC/MS/MS/MS. In this previous work, a 15 pmol/nmol serotonin metabolite ratio cutoff was established in postmortem urine, below which it could be conclusively determined that no recent antemortem ethanol consumption had occurred. In the current study, this newly validated analytical method was applied to five ethanol-positive aviation fatalities where the origin of the ethanol present could not previously be conclusively determined.

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INTRODUCTION

The microbial formation of ethanol in postmortem specimens is by far the most likely complication encountered when examining ethanol results. The first report dealing with postmortem formation of ethanol in corpses appeared in 1936.[1] That report established that postmortem presence of ethanol in itself could not be used as proof of antemortem ingestion. Today, it is known that many different microbes are responsible for postmortem formation of ethanol.[2] Investigations have been performed to identify particular species of bacteria, yeast and/or fungi responsible for ethanol production and the mechanism by which it is formed.[3-6] *Candida albicans* has been the microbe most often ascribed to be responsible for postmortem production of ethanol in humans.[7, 8] This species of yeast is commonly found in humans *in vivo*.[9] However, approximately 100 species of bacteria, yeast, and fungi have been shown capable of producing postmortem ethanol.[3] Glucose is the most prevalent substrate in the human body utilized by ethanol-forming microbes to produce ethanol.[10] Other endogenous compounds can also be utilized as substrates including, but not limited to, lactate, mannitol, galactose, maltose, sucrose, and lactose.[10-13]

Possible exploitation of the metabolism of serotonin as a biological marker for ethanol consumption began to gain considerable interest in the past decade.[14-16] The metabolism of 5-HT initially involves oxidative deamination to form the transitory intermediate, 5-hydroxyindole-3-acetaldehyde (5-HIAL). This intermediate can undergo either oxidation or reduction, as shown in Figure 1. Oxidation of the aldehyde, catalyzed by aldehyde dehydrogenase, leads to formation of 5-hydroxyindole-3-acetic acid (5-HIAA), the predominant metabolite of 5-HT.[16, 17] Reduction, catalyzed by aldehyde reductase, leads to formation of 5-hydroxytryptophol (5-HTOL), usually a relatively minor metabolite of 5-HT.[18] However, ethanol consumption has been shown to lead to a relatively significant enhancement of 5-HTOL production.

An increase in 5-HTOL concentration following ethanol consumption was first reported in 1967.[19] Since that time, it has been clearly demonstrated that consumption of ethanol shifts 5-HT metabolism to promote formation of 5-HTOL and, some reports indicate, to reduce the formation of 5-HIAA.[18, 20-22] Thus, ingested ethanol leads to an elevation in the 5-HTOL/5-HIAA ratio. The 5-HTOL/5-HIAA ratio has been reported to remain elevated for six to eight hours after ingested ethanol has been eliminated from the body.[18, 21, 23] The 5-HTOL/5-HIAA ratio has already been applied to ethanol cessation monitoring programs as a marker of recent ethanol ingestion.[23-25] The 5-HTOL/5-HIAA ratio has also been briefly investigated for possible use with postmortem urine samples.[21]

Historically, concentrations of 5-HTOL and 5-HIAA in individual samples have been measured using two completely different analytical techniques. 5-HIAA concentrations are typically measured at sub-nanomolar concentrations using liquid chromatography with electrochemical detection (LC/EC).[26, 27] 5-HTOL can also be measured by LC/EC, but the detection limits are typically insufficient to measure this compound in most pertinent biological specimens. For that reason, 5-HTOL has routinely been analyzed using gas chromatography with mass spectrometric detection (GC/MS) following derivitization with pentafluoroproprionic acid anhydride.[28] The employment of two different analytical techniques to obtain the 5-HTOL/5-HIAA ratio in a specimen obviously decreases the precision and reliability of the final result. Recently, our laboratory developed a single analytical method for the simultaneous determination of both 5-HTOL and 5-HIAA using liquid chromatography with mass spectrometric detection (LC/MS).[14]

Our laboratory evaluated five ethanol-positive fatalities from separate general aviation accidents in which the source of the ethanol present could not conclusively be attributed to either antemortem ethanol consumption or postmortem microbial ethanol formation. Utilizing this newly developed analytical technique for the determination of the 5-HTOL/5-HIAA ratio, the origin of the ethanol present in each of these cases was investigated.

MATERIALS AND METHODS

Chemicals and Solutions

All aqueous solutions were prepared using double deionized water (DDW), which was obtained using a Milli-QT$_{plus}$ Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). All chemicals were purchased in the highest available purity and used without any further purification. Sodium chloride, sodium acetate, acetic acid, β-glucuronidase,

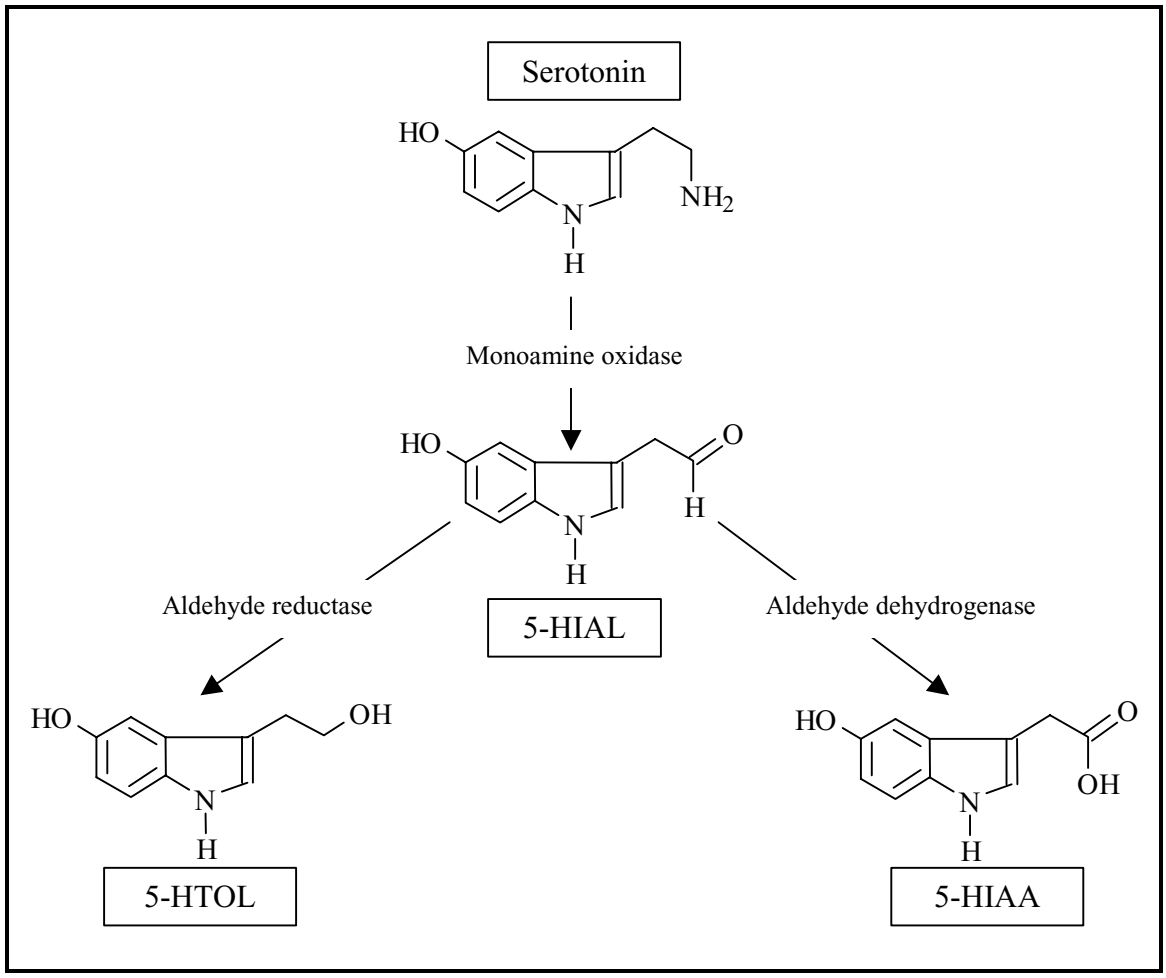

Figure 1. The Metabolism of serotonin.

5-hydroxytryptophol, 5-hydroxyindole-3-acetic acid, and 5-methoxy-2-methyl-3-indoleacetic acid were purchased from Sigma Chemical Company (St. Louis, MO). Methanol, acetonitrile, ammonium hydroxide, hydrochloric acid, ethyl acetate, and nitric acid were purchased from Fisher Scientific (Pittsburgh, PA). Formic acid (97%) was purchased from ICN (ICN Biomedicals, Inc., Irvine, CA). N,O-bis[trimethylsilyl]trifluoroacetamide with 1% trimethylchlorosilane (BSTFA/1%-TMCS) was purchased from Pierce Chemicals (Rockford, IL). All acetate buffers described below were prepared by first making stock solutions of the desired final concentration of both sodium acetate and acetic acid. One of these was then titrated with the other until the desired pH was achieved. The pH of all solutions was measured using a Corning model 430 pH meter (Corning Life Sciences, Acton, MA) with a Corning 3-in-1 model pH electrode.

Two separate 10 mL stock solutions of both 5-HTOL and 5-HIAA were prepared independently at 1.00 mg/mL (5.64 mM 5-HTOL; 5.23 mM 5-HIAA) in methanol. Each of these stock solutions was derived from a unique lot of dry chemical obtained from the manufacturer. These two stock solutions were subsequently identified as calibrators and controls. 5-Methoxy-2-methyl-3-indoleacetic acid (5-MMIA), a compound not known to occur *in vivo*, was used as the internal standard for these experiments and was prepared at a concentration of 100 µg/mL (0.450 mM) in 10 mL of methanol. These indolic compounds are light sensitive, so care was taken to use volumetric flasks wrapped in aluminum foil to prevent photodegradation. Once prepared, the solutions were transferred to 20 mL amber glass bottles, capped, and placed in freezer storage at -20°C. While these solutions are stable for at least 180 days,[14] for maximum assurance of the quality of data, we used no stock solutions that were over 30 days old.

Since 5-HTOL in human-derived specimens is predominately found as the glucuronide derivative, we initially hydrolyzed the samples using the enzyme β-glucuronidase. A solution of β-glucuronidase was prepared

by adding 2.5 mL of pH 5.00, 0.10 mM sodium acetate buffer to 250,000 units of the solid enzyme and mixing to dissolve. This yielded a final concentration of 100,000 units/mL. This solution was stored at -20°C. Like the stock standards, this solution was discarded after storage for a maximum of 30 days. However, it typically was used entirely within 7 days following preparation.

The aqueous portion of the HPLC buffer was 50.0 mM formic acid adjusted to pH 5.00 with conc. ammonium hydroxide. Aqueous buffer and acetonitrile were mixed in a 98:2 ratio, respectively, to help prevent the growth of microbes, and this mixture was filtered through a vacuum filtering apparatus that incorporated a 0.45 µm GH polypro 47 mm hydrophilic, polypropylene membrane filter obtained from Pall Gelman laboratory (Pall Corp., East Hills, NY). The primary organic component of the mobile phase was HPLC grade methanol, which was filtered prior to use through a vacuum filter apparatus that incorporated the same type of membrane filter. The ratio of the previous aqueous mixture to methanol was 20:80 in the final HPLC mobile phase.

Instrumentation

Analyte separation was achieved using a Hewlett Packard 1100 HPLC (Hewlett Packard Co., Wilmington, DE) equipped with a Security Guard™ C-8 guard column (4.0 mm x 3.0 mm i.d., 3 µm particles) from Phenomenex® (Torrance, CA), followed immediately by a Supelcosil™ LC-18 (150 mm x 4.6 mm i.d., 3 µm particles) analytical column from Supelco (Supelco/Sigma-Aldrich, Bellefonte, PA). Samples were injected using a Hewlett Packard G1313A autosampler. Identification and quantitation were accomplished using a Finnigan model LCQ atmospheric pressure chemical ionization (APCI) ion trap mass spectrometer (ThermoFinnigan Corp., San Jose, CA), which utilized nitrogen as the sheath gas and helium as the reagent gas. Control of the HPLC system, integration of the chromatographic peaks, and communication with the mass spectrometer were accomplished using a Gateway 2000 E-4600-SE personal computer using Xcalibur™ LC/MS software (ThermoFinnigan Corp., San Jose, CA).

LC/MS/MS and LC/MS/MS/MS Methods

The analytical method employed for these experiments was described in detail by Johnson *et al.*[14] In brief, the method employed was as follows. For all determinations, the HPLC was operated in an isocratic mode with a flow rate of 1.0 mL/min. The sample injection volume was 10 µL. Detection by the ion-trap mass spectrometer was divided into three unique segments, with each segment dedicated to the detection of a specific compound. Segment 1 was used for analysis of 5-MMIA, segment 2 analyzed for the TMS derivative of 5-HTOL, and segment 3 analyzed for the TMS derivative of 5-HIAA.

Specimen Preparation and Extraction

Calibration curves were prepared by dilution utilizing human certified negative urine as the diluent. Human certified negative urine, as obtained from the manufacturer (UTAK Laboratories Inc., Valencia, CA), is guaranteed to be free of any artificial pharmaceutical compounds and abnormal organic volatiles. Through the course of our initial investigations, this processed urine was also found to be negative for both 5-HTOL and 5-HIAA. The calibrators were prepared from one set of the original stock standard solutions of 5-HTOL and 5-HIAA. Controls were prepared in a similar manner to calibrators, using the same human certified negative urine as diluent, but employing the second set of original stock solutions. Calibrators, controls, and postmortem urine specimens, all simply referred to below as samples, were prepared and extracted in the following manner.

Three mL aliquots of individual samples were transferred to 16 x 150 mm screw-topped culture tubes. To each sample, 1.00 mL of a 1000 ng/mL (4.50 µM) internal standard solution, prepared by dilution of its stock solution with water, was added. β-Glucuronidase solution (7500 units; 75 µL) followed by 1.00 mL of 0.10 mM pH 6.00 sodium acetate buffer was added to each sample. The samples were vortexed briefly and incubated at 70°C for 45 minutes to facilitate hydrolysis of the glucuronide conjugate. In our initial investigations, complete hydrolysis of the 5-HTOL-glucuronide conjugate was achieved after incubation with β-Glucuronidase for 30 minutes at 70°C. However, an incubation time of 45 minutes was chosen to ensure that specimens with extremely elevated 5-HTOL concentrations were also completely hydrolyzed. Following hydrolysis, samples were allowed to cool to room temperature. Then 2.00 mL of a 0.10 M sodium acetate buffer, pH 6.00, and 0.50 mL of a saturated sodium chloride solution were added to each sample, and the tubes were briefly vortexed. Ethyl acetate (9.00 mL) was added to each tube, and the tube was tightly capped. The mixture was then placed on a rotary mixing wheel and gently mixed for 20 minutes by simple rotation of the wheel at 6 rpm. Following mixing, the samples were centrifuged at 820xg for 5 minutes. The organic (upper) layer of each sample was transferred using a disposable pipette to a clean 10 mL conical tube and dried in a water bath at 40°C under a constant stream of nitrogen. Once dryness was achieved, the samples were removed from the evaporator. Both ethyl acetate (50 µL) and BSTFA/1%-TMCS (50 µL) were added to each sample. The tubes were

capped, vortexed briefly, and placed in a heating block at 80°C for 20 minutes. Following derivitization, the tubes were removed from the heating block, allowed to cool to room temperature, and subsequently evaporated to dryness in a water bath at 40°C under a constant stream of nitrogen. The samples were reconstituted in 50 µL of methanol, vortexed briefly, and transferred using a 50 µL pipette to micro-vials for LC/MS analysis.

CASE EXAMINATION

Case #1

A civil aviation accident occurred in the Atlantic Ocean. The wreckage from the accident was located approximately 9 hours after last contact with the victim. The victim, however, was not discovered until reappearing on shore approximately 1.5 months after the accident had occurred. Specimens examined from the victim for volatile organic compounds (VOCs) included blood, urine, skeletal muscle, and brain. Ethanol was present in every tissue and fluid examined from this case. The concentrations of ethanol found in these specimens were 38 mg/dL, 25 mg/dL, 12 mg/hg, and 39 mg/hg, respectively for the four samples mentioned above. The blood and tissue samples were noted as putrefied by visual inspection, and the urine was bloody. Also present in this case were numerous other VOCs, including n-propanol, n-butanol, and acetaldehyde at substantial concentrations. These other VOCs, like ethanol, were present in each of the specimens analyzed. The distribution of ethanol in this case was questionable because: 1) the highest concentration of ethanol detected was in the brain, which has a lower water content than both blood and urine and, therefore, would normally be expected to contain lower concentrations of ethanol, and 2) the blood ethanol concentration considerably exceeded the urine ethanol concentration, which is possible, but is not common in aviation fatalities. The presence of numerous other VOCs also supported the possibility that the ethanol present in this case resulted from postmortem microbial formation and not antemortem consumption. By LC/MS analysis, the urinary 5-HTOL/5-HIAA ratio for this case was determined to be 2.1 pmol/nmol. This value is well below the cutoff of 15 pmol/nmol established for antemortem ethanol consumption[14, 23, 29], supporting the conclusion that recent ethanol consumption had not occurred prior to the accident.

Case #2

A civil aviation accident occurred in the Pacific Ocean near Alaska. The body of the victim was recovered 17 days after the accident. The specimens initially examined for ethanol and other VOCs were blood and urine. These specimens contained ethanol at concentrations of 54 mg/dL and 9 mg/dL respectively. The distribution of ethanol in this case raises immediate questions as to its source. However, the low urinary ethanol concentration, when compared to the blood value, may be the result of dilution of the urine due to the length of time the victim was submerged in the ocean. Analysis of other VOCs present in the victim was limited to only acetaldehyde, which was present at 12 mg/dL in the blood. This concentration of acetaldehyde result could reasonably be considered to support either antemortem consumption or postmortem microbial formation. To provide a more definitive answer to the ethanol origin question, we examined the 5-HTOL/5-HIAA ratio for this case. The ratio was determined to be 3.2 pmol/nmol in urine. This value is well below the cutoff established for recent antemortem ethanol consumption, leading to the conclusion that microbes were responsible for the formation of the ethanol present in the blood of this victim.

Case #3

A civil aviation accident occurred in the Pacific Ocean near Southern California. The pilot's body was recovered from the ocean at a depth of approximately 70 feet, 16 days following the accident. Specimens initially analyzed for volatile organic compounds in this case were blood, urine, brain, and skeletal muscle. The blood and urine both contained ethanol at concentrations of 22 and 18 mg/dL, respectively. Brain and skeletal muscle were found to contain ethanol at concentrations of 27 and 9 mg/hg, respectively. The distribution of ethanol in these fluids and tissues, although not completely consistent with ethanol ingestion, does not exclude a conclusion of antemortem ethanol consumption. Various volatiles including acetaldehyde, n-propanol, n-butanol, and acetone were also present in these specimens, which indicates postmortem microbial activity. This case represents another classic example of inconclusive ethanol origin. Without further investigation it would be difficult to conclusively determine whether the pilot had recently consumed ethanol. Utilizing our newly validated method, we found the 5-HTOL/5-HIAA ratio to be 0.41 pmol/nmol in urine. This value is considerably below the 15 pmol/nmol cutoff, thus strongly indicating the absence of recent antemortem ethanol ingestion. The presence of ethanol in this case was thus reported as resulting from postmortem microbial formation.

Case #4

A civil aviation accident occurred in a rural region of Northern Michigan. The accident was witnessed by numerous people, which resulted in a quick response by emergency personnel and the prompt recovery of the victim. Specimens initially analyzed for VOCs were

blood, urine, vitreous humor, brain, and skeletal muscle. The blood, urine, and vitreous humor contained ethanol at concentrations of 17, 25, and 25 mg/dL, respectively. Brain and skeletal muscle were found to contain ethanol at concentrations of 146 and 170 mg/hg, respectively. The distribution of ethanol in the fluids initially examined from this case is fully consistent with antemortem ethanol consumption. The tissues examined, however, contained ethanol completely inconsistent with the fluid results. Three other VOCs were detected in these specimens including n-propanol, acetaldehyde, and n-butanol. The concentrations of the three VOCs were significantly higher in brain and muscle than those in the fluids examined. For example, n-propanol was detected at 14 mg/hg in the brain but only 1 mg/dL in the blood and urine; and n-butanol was detected at 19 mg/hg in the muscle but only 1 mg/dL in the blood and the urine. Upon specimen collection by the medical examiner in aviation fatalities, blood specimens are obtained and stored in sealed tubes that contain a preservative, while urine and vitreous humor are collected in sealed tubes that contain no preservative.[30] Additionally, tissue specimens are placed in plastic zip-top bags, also without addition of preservative. When our laboratory received this case it was noted that the temperature of the container carrying the specimens in questions was 10°C, which is above the desired transport temperature of 4°C. It was also noted by visual inspection that the tissue specimens were putrefied, and they had a foul odor. It is probable that the tissue specimens in this case were contaminated with microbes either before or during collection and that these microbes had sufficient time to produce substantial quantities of ethanol.

This case represents one of the most difficult ethanol origin scenarios possible. It appears upon initial examination that ethanol was consumed antemortem. Then as a result of trauma experienced during the accident, microbes contaminated both brain and muscle specimens. Ethanol formation subsequently occurred while these tissue specimens were in transit. We applied our LC/MS method to the urine available from this case and found the 5-HTOL/5-HIAA ratio to be 67 pmol/nmol. This value is well above the established 15 pmol/nmol cutoff, thus confirming the recent consumption of ethanol prior to death as suggested by the fluid ethanol distributions.

Unlike the interpretation of a 5-HTOL/5-HIAA ratio below 15 pmol/nmol, which conclusively eliminates the possibility of recent ethanol consumption, caution must be used when interpreting a 5-HTOL/5-HIAA ratio above the 15 pmol/nmol cutoff. In antemortem experiments, it has been demonstrated that the 5-HTOL/5-HIAA ratio may remain elevated above the 15 pmol/nmol cutoff value for some 2 - 8 hrs after all consumed ethanol has been eliminated from the body.[18, 23] Therefore, there remains a possibility that all of the ethanol present in this case was produced by postmortem microbial formation, and the elevated 5-HTOL/5-HIAA ratio was due to ethanol that had been consumed within the previous 8 - 12 hrs but completely eliminated from the body prior to the fatal accident.

Case #5

A civil aviation accident occurred in a rural region of California. The accident site was not discovered until 7 days following the crash, and the victim was found submerged in a river. Specimens initially analyzed for VOCs in this case included blood, urine, skeletal muscle, and brain. These specimens each contained ethanol at concentrations of 31 mg/dL, 48 mg/dL, 49 mg/hg, and 57 mg/hg, respectively. The distribution of ethanol in the fluids and tissues examined from this case, although not considered normal, does not immediately rule out the possibility of antemortem ethanol ingestion.[31] Other VOCs were detected in these specimens as well. The volatiles found were n-propanol in each specimen, acetaldehyde in blood and urine, n-butanol in each specimen and isopropanol in blood, urine, and skeletal muscle. As previously discussed, the detection of other VOCs in the specimen of interest indicates postmortem microbial activity and, therefore, the possibility of postmortem ethanol formation.

This case represents another difficult question of ethanol origin. The classic ethanol origin indicators could not conclusively rule out the possibility of antemortem ethanol ingestion. We applied the LC/MS method to the urine available from this case and found the 5-HTOL/5-HIAA ratio to be 0.08 pmol/nmol. This value is well below the 15 pmol/nmol cutoff, thus confirming no recent antemortem ethanol ingestion had occurred, but instead, indicated the postmortem microbial formation of ethanol in this case.

DISCUSSION

Two classic indicators are commonly employed by forensic laboratories to discern the origin of ethanol in postmortem cases. These indicators are: (1) the distribution of ethanol between various bodily fluids and tissues from a single victim and (2) the presence of additional VOCs in the bodily fluids and tissues of the victim. During life, ethanol distributes fairly rapidly throughout the body according to the water content of the various fluids and tissues.[31, 32] Postmortem microbial ethanol formation alters the ethanol concentration in the examined fluids and tissues and consequently disrupts the usual distribution pattern of ethanol. By comparing ethanol results obtained from two or more fluids and tissues from one

case, one can make an informed assumption concerning the origin of any ethanol present. The presence of other volatiles such as acetaldehyde, acetone, sec-butanol, isopropanol, n-propanol, methanol, and n-butanol in an ethanol-positive specimen suggests microbial action and, therefore, possible postmortem ethanol formation. The lack of other commonly analyzed volatiles suggests the absence of microbial postmortem activity but does not exclude the possibility of postmortem ethanol formation.[3, 8, 33-38]

Furthermore, urine was once considered immune to microbial ethanol formation because it is sealed in the bladder and not normally exposed to microbes and/or a substrate such as glucose for the microbes to consume. However, this view of urine has been recently questioned.[4, 12, 39-41] It is now known that postmortem microbial production of ethanol in urine is quite feasible and, indeed, under the circumstances of a fatal aviation accident may be likely. After careful application of the above-mentioned ethanol origin indicators to the five cases in question, the precise source of ethanol origination was still unclear. Therefore, application of the newly validated LC/MS method[14] for determination of the serotonin metabolite ratio was undertaken.

A 5-HTOL/5-HIAA ratio below 15 pmol/nmol clearly eliminates the possibility of recent ethanol consumption prior to death, while a 5-HTOL/5-HIAA ratio above 15 pmol/nmol indicates recent ethanol ingestion. It must be stressed, however, that in an ethanol positive case with a 5-HTOL/5-HIAA ratio above 15 pmol/nmol, there remains a possibility that the ethanol present in the specimen originated from postmortem microbial formation, and not consumption. This possibility arises from the time lag between complete ethanol elimination from the body and the return of the 5-HTOL/5-HIAA ratio to normal levels. In fact, the 5-HTOL/5-HIAA ratio can remain elevated above normal for 2-8 hours after ingested ethanol has been completely eliminated from the body.[21, 42] Further, chemically identical ethanol resulting from postmortem microbial formation can be superimposed upon any residual ethanol from recent consumption.

The cases represented in this paper exemplify the usefulness of employing the 5-HTOL/5-HIAA ratio to determine if recent ethanol consumption had occurred. We suggest that the relative simplicity of this procedure should make the 5-HTOL/5-HIAA ratio methodology more readily applicable to the routine assessment of antemortem versus postmortem origin of ethanol found in postmortem fluid and tissue samples. The application of this method is also notably not limited to postmortem samples. Indeed, contested ethanol results from routine screenings in various drug programs, and criminal cases could readily be evaluated through the use of this procedure.

CONCLUSIONS

A newly developed LC/MS procedure for the analysis of 5-HTOL and 5-HIAA and the associated 5-HTOL/5-HIAA ratio was applied to five ethanol-positive civil aviation fatalities where ethanol origin could not previously be determined. The application of this method has demonstrated the utility of this approach in assessing ethanol origin in postmortem specimens. One of the most important aspects of this novel method is the simultaneous analysis of both compounds using a single extraction method and a single analytical technique. This greatly increases the precision in the methodology and, thus, the certainty of the subsequent conclusions.

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